Investigation of the Effect of Environmental Conditions on the Rate of Exopolysaccharide Synthesis in *Azotobacter vinelandii*

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Exopolysaccharide was produced by *Azotobacter vinelandii* NCIB 9068 grown under a variety of environmental conditions. The rate of exopolysaccharide synthesis from sucrose in continuous culture remained within the range 5 to 14 mg-atom C (g cell)⁻¹ h⁻¹ for specific growth rates of 0.05 to 0.25 h⁻¹, for rates of sucrose utilization of 16 to 105 mg-atom C (g cell)⁻¹ h⁻¹ and for a range of growth-limiting nutrients, including sucrose.

INTRODUCTION

A wide range of bacteria are known to produce copious quantities of exopolysaccharide, commonly manifest as large mucoid colonies on agar plates or highly viscous broths in submerged culture. This property is particularly common amongst plant pathogens such as *Xanthomonas* (Dye & Lelliott, 1974), *Erwinia* (Eden-Green & Knee, 1974) and the aerobic nitrogen-fixing bacteria *Rhizobium* (Jordan & Allen, 1974), *Beijerinckia* (Becking, 1974) and *Azotobacter* (Johnstone, 1974). The exopolysaccharide can form a large proportion of the biosynthetic products of the bacteria; for instance, ratios of exopolysaccharide to cell dry weight of 3 have been reported for *Azotobacter* (Deavin *et al.*, 1977) and 1 for *Rhizobium meliloti* (Dudman, 1964). The functions of exopolysaccharides in nature have not been established, though it has been suggested that they may protect against desiccation, phagocytosis and phage attack (Wilkinson, 1958; Sutherland, 1972) or high oxygen tension (Postgate, 1974), be involved in uptake of metal ions (Wilkinson, 1958; Sutherland, 1972), act as adhesive agents (Johnson *et al.*, 1977) or ATP sinks (Neijssel & Tempest, 1976) or participate in interactions between plants and bacteria (Dazzo & Brill, 1977; Morris *et al.*, 1977).

As part of an investigation of the physiology of exopolysaccharide production by *Azotobacter vinelandii* we have studied its synthesis under a range of nutritional stresses. In addition, by varying growth rate and respiration we have investigated the relationship between flux of carbohydrate into the cell and the flux through the main catabolic pathways and exopolysaccharide biosynthetic pathway. The exopolysaccharide produced by this bacterium is a partially acetylated (1→4)-linked linear copolymer of L-mannuronic acid and D-guluronic acid (Gorin & Spencer, 1966; Larsen & Haug, 1971) and is thus similar to the alginates produced by certain species of brown seaweed.

METHODS

The organism, its maintenance and growth media. *Azotobacter vinelandii* (NCIB 9068) was maintained on growth medium I solidified with 1.5 % (w/w) agar. Stock cultures were subcultured every 6 months, grown at 30 °C for 2 d and stored at 4 °C. Working cultures were subcultured from the stock culture every 6 months and were themselves subcultured monthly.

Two growth media were used. Medium I contained (per litre): sucrose, 20 g; KH_2PO_4 , 8 mg; K_2HPO_4 , 32 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NaCl , 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 42 mg; Na_2MoO_4 , 1 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mg; H_3BO_3 , 2.9 mg; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.09 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mg. All ingredients were autoclaved together for 1 h at 0.1 MPa. Medium II contained (per litre): sucrose, 40 g; KH_2PO_4 , 64 mg; K_2HPO_4 , 0.26 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 g; NaCl , 1.6 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 8 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.34 g; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 17 mg; H_3BO_3 , 23 mg; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.72 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 9.6 mg. To avoid precipitation on autoclaving, the medium was separated into two batches which were prepared as follows to give 20 l of complete medium. Batch A contained sucrose, KH_2PO_4 and K_2HPO_4 in 14 l, to which MgSO_4 , NaCl , Na_2MoO_4 and trace elements other than CaCl_2 and FeCl_2 were added in 4 l after autoclaving separately. Batch B contained CaCl_2 autoclaved in 1900 ml, to which FeCl_2 in 100 ml was added after filter sterilization. Medium batches A and B were added to the culture through separate pumps and tubing in the ratio 9:1. The combined addition rates were adjusted to give the desired dilution rate. To obtain growth limitation by the appropriate nutrient, the following modifications were made to medium II (per litre): molybdenum limitation, no Na_2MoO_4 added; phosphate limitation, 32 mg KH_2PO_4 and 65 mg K_2HPO_4 ; iron limitation, 1.7 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$; carbon limitation, 8 g sucrose; calcium limitation, no $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ added; potassium limitation, 52 mg KCl , 57 mg NaH_2PO_4 and 0.215 g Na_2HPO_4 added, and KH_2PO_4 and K_2HPO_4 omitted; sulphate limitation, 40 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.32 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

Continuous culture apparatus and conditions. The organisms were continuously cultured in a mechanically stirred 1 litre culture vessel attached to a type 1/1000 laboratory fermentation module (L.H. Engineering, Stoke Poges, Buckinghamshire). In certain experiments a draught tube was inserted into the vessel, but in others, as indicated, this was replaced by four baffles (1×13 cm) fitted symmetrically around the perimeter of the vessel. The fermentation temperature was 30 °C. The pH was maintained at 7.4 (± 0.1) by automatic addition of 1 M-NaOH. Foaming was controlled by addition, as necessary, of silicone antifoaming agent (BDH). The rate of air flow into the culture and impeller speed were varied as indicated. Oxygen uptake and carbon dioxide evolution were monitored continuously with a paramagnetic oxygen analyser (type OA 184, Taylor Servomex, Crowborough, TN6 3DV, Sussex) and an infrared gas analyser (model SB2, Grubb Parsons, Walkergate, Newcastle NE6 2YB). Following a change in conditions a steady state was considered to exist when A_{540} and oxygen uptake had remained constant for at least three residence times. Growth limitation by a medium constituent was demonstrated by (i) obtaining an increase of at least 10 % in A_{540} on increasing the concentration of a nutrient supplied to the culture and (ii) obtaining a steady state of higher cell mass using unmodified medium II as shown in Table 1. Oxygen limitation was obtained by decreasing the impeller speed and thus the rate of gas transfer into the sample. Nitrogen limitation was obtained by supplying the culture with argon containing oxygen (20 %, v/v) at 950 ml min⁻¹ and air at 50 ml min⁻¹.

Cell dry weight determination. A mixture of culture (40 ml), 5 M-NaCl (0.8 ml) and 0.5 M-Na₂EDTA (0.8 ml) was centrifuged at 24000 g for 40 min. The supernatant obtained was decanted, and the sediment was resuspended in distilled water and centrifuged at 24000 g for 20 min. The final sediment was transferred to a preweighed porcelain dish and dried to constant weight at 105 °C.

Polysaccharide determination. The supernatant (25 ml) obtained from the first centrifugation described above was added to propan-2-ol (75 ml) and the mixture was shaken vigorously. After 10 min, the precipitate obtained was filtered on to a predried and preweighed Whatman GF/A filter disc and washed with 100 ml of propan-2-ol/water (3:1, v/v). The filter disc plus precipitate was dried under vacuum at 45 °C for 24 h, reweighed and the concentration of polysaccharide in the culture broth was calculated.

Poly-3-hydroxybutyrate was determined by the method of Law & Slepecky (1961).

Sucrose was determined as its trimethylsilyl ether derivative by gas-liquid chromatography using trehalose as an internal standard (Sweeley *et al.*, 1963).

RESULTS

Variation of growth-limiting nutrient

Nutrient limitations on growth in the chemostat were demonstrated as shown in Table 1. Exopolysaccharide was produced under all limitations tested (Table 2). With the exception of oxygen-limited conditions, there was little or no poly-3-hydroxybutyrate (PHB) produced and the rate of exopolysaccharide synthesis (q_{ps}) was between 5.0 and 10.5 mg-atom C (g cell)⁻¹ h⁻¹. Oxygen limitation resulted in a significantly lower q_{ps} when based on whole cell mass, but microscopic examination showed that much of the cell mass was poly-3-hydroxybutyrate. The q_{ps} based on active biomass would have been considerably higher. When sucrose, the carbon and energy source, limited growth, exopolysaccharide was produced at a rate similar to that obtained with most other limitations. Under this condition the

Table 1. *Demonstration of nutrient limitations*

Limiting nutrient	A_{540} before limitation test*	Change in conditions for limitation test	A_{540} after change in conditions*†
MoO_4^{2-}	0.056	Inflowing medium changed to complete medium II	0.071 (7 h) 0.18 (18 h)
PO_4^{3-}	0.17	Inflowing medium changed to complete medium II	0.34 (24 h)
Fe^{2+}	0.105	$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ concn in inflowing medium increased from 1.7 to 5.2 mg l ⁻¹	0.18 (6 h)
C (sucrose)	0.117	Inflowing medium changed to complete medium II	0.163 (17 h) 0.245 (24 h)
N (N_2)	0.20	Air in Ar/ O_2 /air mixtures increased from 5 to 10 %	0.26 (7 h)
Ca^{2+}	0.088	Inflowing medium changed to one containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at 4 mg l ⁻¹	0.26 (23 h)
SO_4^{2-}	0.11	Inflowing medium changed to complete medium II	0.27 (24 h)
O_2	0.14	Impeller speed increased from 400 to 600 rev. min ⁻¹	0.58 (24 h)

* Culture broth was diluted 1 in 10 with water before A_{540} measurements.

† Times of measurement after change in conditions are indicated in parentheses.

Table 2. *Exopolysaccharide production under a variety of nutrient limitations*

Culture conditions were as described in Methods. The fermentation vessel was fitted with baffles instead of a draught tube. Growth medium II was used with the stated variations made in order to obtain the desired growth limitation. Cultures grown on unmodified medium II were phosphate-limited and had a steady state dry cell mass of 4 g l⁻¹. Respiration rate, q_{CO_2} was maintained within the range 15 to 30 mmol CO_2 (g cell)⁻¹ h⁻¹ [except in the O_2 -limited condition where it was 8 mmol CO_2 (g cell)⁻¹ h⁻¹] by adjusting the fermenter impeller speed. The gas flow into the culture was 1 l min⁻¹; the dilution rate was 0.15 h⁻¹.

Limiting nutrient	Cell mass (g l ⁻¹)	Polysaccharide produced [mg-atom C (g cell) ⁻¹ h ⁻¹]
MoO_4^{2-}	1.1	10.5
PO_4^{3-}	1.9	8.9
Fe^{2+}	1.4	8.1
C (sucrose)	1.3	7.7
N (N_2)	1.5	6.8
Ca^{2+}	1.2	6.2
SO_4^{2-}	1.7	7.8
K^+	1.9	5.0
O_2	1.2	1.9

bacteria might have been expected to make the most efficient use possible of their available carbon and energy substrate.

Variation of dilution rate

The relationship between q_{ps} and the specific growth rate (μ) of *A. vinelandii* was investigated in continuous culture under phosphate-limited conditions. Cell mass was constant at dilution rates between 0.15 and 0.25 h⁻¹ (Table 3) but increased at lower dilution rates due to intracellular accumulation of poly-3-hydroxybutyrate. In the dilution rate range 0.05 to 0.25 h⁻¹, q_{ps} was between 10.6 and 14 mg-atom C (g cell minus g PHB)⁻¹ h⁻¹ being largely independent of specific growth rate. The rate of sucrose utilization (q_s) varied from 35 to 98 mg-atom C (g cell minus g PHB)⁻¹ h⁻¹ over the same range of growth rates and was determined mainly by the respiration rate, q_{CO_2} . In Table 3, the partition of carbon between cell synthesis, respiration and exopolysaccharide synthesis is shown.

Table 3. *Fate of carbon assimilated by A. vinelandii as a function of dilution rate in phosphate-limited culture*

Continuous culture conditions were as described in Methods. Growth medium I was used. The impeller speed was 700 rev. min⁻¹, the air flow was 1.2 l min⁻¹ and a draught tube was inserted into the fermentation vessel. For calculation of mg-atom C of exopolysaccharide, the carbon content of this partially acetylated sodium polyuronide was taken as 37 %.

<i>D</i> (h ⁻¹)	Cell mass (g l ⁻¹)	Cellular PHB (g l ⁻¹)	Fate of carbon assimilated [mg-atom C (g cell minus g PHB) ⁻¹ h ⁻¹]			
			Sucrose used	Cell mass produced	CO ₂ produced	Exopolysaccharide produced
0.05	1.37	0.86	49	5.8	35	10.6
0.10	0.69	0.07	42	4.7	23	11.5
0.15	0.44	0.01	56	6.3	33	14
0.20	0.43	< 0.01	98	8.4		13
0.25	0.45	< 0.01	35	10.5	13	13.3

Table 4. *Sucrose use and exopolysaccharide production as a function of respiration rate in phosphate-limited culture*

Culture conditions were as described in Methods. The dilution rate was 0.15 h⁻¹, the fermentation vessel was fitted with a draught tube and the air flow was 1.2 l min⁻¹. Growth medium I was used. Cellular poly-3-hydroxybutyrate content was less than 5 % of the cell concentration. For calculation of mg-atom C of exopolysaccharide, the carbon content of this partially acetylated sodium polyuronide was taken as 37 %.

Impeller speed (rev. min ⁻¹)	Cell mass (g l ⁻¹)	Fate of carbon assimilated [mg-atom C (g cell) ⁻¹ h ⁻¹]			
		Sucrose used	Cell mass produced	CO ₂ produced	Exopolysaccharide produced
400	0.55	16	6.3	6.4	6.4
500	0.42	34	6.3	12	6.9
600	0.44	40	6.3	28	11.6
700	0.44	56	6.3	33	12.1
870	0.49	105	6.3	71	8.4

Variation of respiration rate

Further information on the relationship between the flux of hexose entering the exopolysaccharide biosynthetic pathway and central metabolic pathways was sought by investigating exopolysaccharide synthesis at a range of q_{CO_2} values with phosphate-limited growth conditions. q_{CO_2} was manipulated by adjusting the fermenter impeller speed and thereby altering the rate of oxygen transfer. As reported previously (Dalton & Postgate, 1969), q_{CO_2} increased with increased oxygen availability. Cell mass was essentially independent of respiration rate. q_{PS} varied less than twofold [from 6.4 to 12.1 mg-atom C (g cell)⁻¹ h⁻¹] for a tenfold increase in q_{CO_2} (Table 4). q_{S} increased sixfold over this q_{CO_2} range. These findings indicate that the flux of hexoses into the exopolysaccharide biosynthetic pathway is relatively independent of the flux of substrate into the cell and that of hexoses into the central metabolic pathways.

DISCUSSION

Batch-culture studies on *Escherichia coli* and *Klebsiella aerogenes* (Duguid & Wilkinson, 1953; Wilkinson, Duguid & Edmunds, 1954) and subsequent continuous culture studies on *K. aerogenes* (Neijssel & Tempest, 1975) and a *Pseudomonas* sp. (Williams, 1975) indicated that exopolysaccharide production occurred under conditions where excess carbohydrate was present, growth being limited by a nutrient other than the carbon and energy source.

In the continuous culture studies, the highest rates of exopolysaccharide synthesis were observed during nitrogen-limited growth. It was suggested that in *K. aerogenes* exopolysaccharide was an 'overflow metabolite', its biosynthesis possibly functioning as a 'slip reaction', effecting the hydrolysis and turnover of ATP (Neijssel & Tempest, 1975, 1976). We have studied exopolysaccharide synthesis by a strain of *A. vinelandii* which excretes partially acetylated alginic acid (Gorin & Spencer, 1966). Most species of *Azotobacter* and other N_2 -fixing aerobes accumulate copious quantities of extracellular polysaccharides. The function of these polymers is not known and strains that have lost the ability to synthesize them are viable under laboratory conditions (Sutherland, 1972). The organisms used in this study synthesized substantial quantities of alginic acid at similar rates under a wide range of conditions. Polysaccharide production under carbon (sucrose) limitation at rates similar to those found for other limiting substrates argues against an 'overflow' mechanism of the type described by Holme (1957) and Neijssel & Tempest (1975) operating in this instance. Production of acetylated alginic acid is also known to occur under carbon (glucose) limitation in mucoid strains of *Pseudomonas aeruginosa* (F. A. Mian, personal communication). In experiments in which the relative rates of flux of carbon for cell biosynthesis, respiration and polysaccharide production were varied between wide limits, the absolute rate of exopolysaccharide synthesis was more or less constant. No inference can be made from these observations on the control system by which the constant rate is achieved. The biosynthetic route to exopolysaccharide diverges from the main catabolic pathways at the hexose phosphate level (Pindar & Bucke, 1974). As mannosephosphate isomerase provides mannose 6-phosphate for alginate synthesis some control might be expected at that level. It has been suggested that competition for polyisoprenoid cofactor between exopolysaccharide synthesis and cell wall synthesis might exist (Sutherland, 1977). The independence of rate of exopolysaccharide synthesis and specific growth rate in *A. vinelandii* is not consistent with such competition.

Our observation that exopolysaccharide production in *A. vinelandii* is unaffected by most environmental conditions was considered surprising, but re-examination of other reports of polysaccharide synthesis suggests that this might not be uncommon. Reported data indicate that the specific rate of exopolysaccharide synthesis in nitrogen-limited continuous cultures of *Xanthomonas campestris* (Silman & Rogovin, 1972) and of *Pseudomonas* sp. (Williams, 1975) varied little for specific growth rates of 0.05 to 0.2 h⁻¹ and 0.025 to 0.25 h⁻¹, respectively (Deavin *et al.*, 1977). Although the physiological significance of these polymers is obscure, the diversion of large proportions of the assimilated carbohydrate into exopolysaccharide in a variety of physiological conditions and the widespread occurrence of exopolysaccharide synthesis in certain groups of bacteria suggest a major role *in vivo*.

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